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(54) Title: ASSAYS FOR *MYCOBACTERIUM TUBERCULOSIS* USING MONOSPECIFIC ANTIBODIES

(57) Abstract

Monospecific antibodies which selectively bind to *Mycobacterium tuberculosis* are described. The monospecific antibodies include monoclonal antibodies and monospecific polyclonal antibodies. Methods of detecting the presence of *Mycobacterium tuberculosis* using monospecific antibodies are described. The assay methods include the use of flow-through immunoassay test devices.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Title: ASSAYS FOR *MYCOBACTERIUM TUBERCULOSIS* USING MONOSPECIFIC ANTIBODIES

ASSAYS FOR *MYCOBACTERIUM TUBERCULOSIS* USING MONOSPECIFIC ANTIBODIES

Field of the Invention

The present invention relates to antibodies which bind to *Mycobacterium tuberculosis* while showing little cross-reactivity with other *Mycobacterium* species, and to assays for the detection of *Mycobacterium tuberculosis* using these antibodies.

Background of the Invention

Tuberculosis is a necrotizing bacterial infection which can be caused in man by two species of tubercle bacilli: *Mycobacterium tuberculosis* and *Mycobacterium bovis*. By far the greatest number of tuberculosis cases in the United States are caused by *M. tuberculosis*. The lungs are most commonly affected, although infection may occur in the kidneys, bones, lymph nodes, meninges, or may be disseminated throughout the body. Confirmation of active *M. tuberculosis* infection requires the identification of the bacillus from tissue or body fluid. A sustained course of chemotherapy is the primary form of treatment for *M. tuberculosis* infection.

Several other species of mycobacteria may cause chronic infections, including *M. avium/intracellulare* and *M. kansasii*.

Conventional methods available for the identification of mycobacteria combine the cultivation of mycobacteria on specific media and the use of differential tests, such as niacin production, nitrate reductase activity, and Tween 80 hydrolysis. *See* Tsukamura, *Rev. Infect. Dis.*, 3, 841 (1981). Radiometric identification methods are also available, such as automated identification using ^{14}C-labeled palmitic acid. *See, e.g.*, Siddiqi, et al., *Amer. Rev. Respir. Dis.*, 130, 634 (1984); Kirihara et al. *J. Clin. Microbiol.*, 22, 841 (1985); Middlebrook et al., *Am. Rev. Respir. Diseases*, 115, 1066 (1977). More recent methods are based on hybridization of radiolabelled DNA probes.

Polyclonal antibodies to mycobacteria have been used to detect mycobacterial antigens in sputum and cerebrospinal fluids. *See, e.g.*, Watt *et al.*, *J. Infect. Dis.*, 158, 681 (1988); Yanez *et al.*, *J. Clin. Microbiol.*, 23, 822 (1986).

Summary of the Invention

A first aspect of the present invention is a monospecific antibody selected from the group consisting of: **(a)** a monoclonal antibody produced by the cell line Mtb-b5.3.5; **(b)** a monoclonal antibody which binds to the 65 kilodalton protein of *Mycobacterium tuberculosis* bound by the monoclonal antibody of (a); **(c)** a monoclonal antibody which binds to the epitope bound by the monoclonal antibody of (a); and **(d)** a polyclonal antibody which binds to the epitope bound by the monoclonal antibody of (a). These monospecific antibodies exhibit essentially no binding to

Mycobacterium avium under the same conditions where they bind to *Mycobacterium tuberculosis*.

A further aspect of the present invention is a monoclonal antibody selected from the group consisting of (a) a monoclonal antibody produced by the cell line Mtb-b5.3.5; (b) a monoclonal antibody which binds to the 65 kilodalton protein of *Mycobacterium tuberculosis* bound by the monoclonal antibody of (a); and (c) a monoclonal antibody which binds to the epitope bound by the monoclonal antibody of (a). These monoclonal antibodies exhibit essentially no binding to *Mycobacterium avium* under the same conditions where they bind to *Mycobacterium tuberculosis*.

Further aspects of the present invention are a monoclonal antibody produced by the cell line Mtb-b5.3.5, the cell line Mtb-b5.3.5, and a cell line producing the monoclonal antibodies described above.

A further aspect of the present invention is a method of assaying the presence of *Mycobacterium tuberculosis* in a sample. In this method a sample is contacted with an antibody as described above under conditions permitting the antibody to bind to *M. tuberculosis*, and then the presence or absence of the antibody bound to the protein is detected. The assay may be either heterogenous or homogenous, and is preferably homogenous.

A further aspect of the present invention is a kit for assaying *Mycobacterium tuberculosis* in a sample, using an antibody as described above conjugated to a detectable group. A further aspect of the present invention is a kit for assaying *Mycobacterium tuberculosis* in a sample, including an antibody as described above and a specific binding partner for the antibody conjugated to a detectable group.

The foregoing and other objects and aspects of the present invention are explained in detail in the specification set forth below.

Brief Description of the Drawings

FIGURE 1 shows the results of specificity testing of candidate monoclonal antibody Mtb-b5 with a variety of *Mycobacterium* species adsorbed on the surface of a microtiter plate, by enzyme-linked immunosorbent assay (ELISA).

FIGURE 2A shows specificity testing of clone Mtb-b5.3 obtained from spent media on a variety of *Mycobacterium* species and strains.

FIGURE 2B shows specificity testing of clone Mtb-b5.3 obtained from ascites on a variety of *Mycobacterium* species and strains.

FIGURE 2C shows specificity testing of clone Mtb-b5.3.5 obtained from ascites on a variety of *Mycobacterium* species and strains.

FIGURE 3 shows the interpolation of molecular weight standards on Western Blot to determine the molecular weight of the protein antigen bound by Mtb-b5.3.5.

FIGURE 4 Results of a dipstick format for the detection of *M. tuberculosis* using monoclonal antibody Mtb-b5.3.5 and dilutions of heat-killed *M. tuberculosis* (strain H37Rv) (striped bar) and *M. kansasii* (strain 714) (solid bar) cells, with goat α anti-Mouse IgG liposomes containing sulforhodamine red for staining.

Detailed Description of the Invention

Monoclonal antibodies (MAbs) have previously been raised against *M. tuberculosis* and other mycobacteria. Such MAbs have been used to identify cultured *M. avium* and *M. tuberculosis* in an immunofluorescence test and in

immunoblots. Kolk et al., *Health Coop. Papers*, 7, 101 (1988); Verstijnen *et al.*, In: Kager and Polderman (eds.), XIIth International Congress for Tropical Medicine and Malaria, Elsevier Science Publishers, Amsterdam (1988). An enzyme-linked immunosorbent assay (ELISA) has been described for the identification, from early cultures, of the *M. tuberculosis* complex, *M. avium* complex, and *M. kansasii*. Schöningh *et al.*, *J. Clin. Microbiol.*, 28, 708 (1990). *See also*: Hewitt et al., *J. Immunol. Methods*, 55, 205 (1982); Ivanyi et al, In: Monoclonal Antibodies Against Bacteria, Vol. 1, Academic Press (1985), at 59-90; Wright et al., In: Swaminathan & Prakesh (Eds.) Nucleic Acid and Monoclonal Antibody Probes, Marcel Dekker, Inc., New York (1989), at 517-556; European Patent Application EP 87 11 8904; WO Patent Application WO 86/02357.

To be useful in the clinical diagnosis of *M. tuberculosis*, an assay must reliably detect the presence of *Mycobacterium tuberculosis* while not cross reacting significantly with *M. kansasii*, *M. scrofulaceum*, *M. gordonae*, *M. avium*, *M. intracellulare*, *M. fortuitum* or *M. chelonae*, as these are organisms commonly isolated in the clinical environment and are similar to *M. tuberculosis*.

As used herein, sensitivity refers to the ability of an assay to reliably detect the presence of *Mycobacterium tuberculosis* (i.e., to give a high percentage of true positive reactions and a low percentage of false negative reactions). As used herein, specificity refers to the ability of an assay to reliably not react with mycobacterial species other than *M. tuberculosis* (i.e., to give a low percentage of false positive reactions and a high percentage of true negative reactions).

Monospecific antibodies of the present invention preferably bind strongly to one or more *M. tuberculosis* strains with little binding, under the same conditions, to any *M. avium*. More preferably, monospecific antibodies of

the present invention preferably bind strongly to one or more *M. tuberculosis* strains with little binding, under the same conditions, to anyone of *M. avium, M. intracellulare. M. gordonae, M. fortuitum, M. smegmatis, M. szulgai, M. xenopi, M. phlei, M. terrae, M. gastri, M. scrofulaceum, M. kansasii,* and *M. chelonae.* More preferably, monospecific antibodies of the present invention preferably bind strongly to one or more *M. tuberculosis* strains with little binding, under the same conditions, to any combination of *M. avium, M. intracellulare. M. gordonae, M. fortuitum, M. smegmatis, M. szulgai, M. xenopi, M. phlei, M. terrae, M. gastri, M. scrofulaceum, M. kansasii,* and *M. chelonae.*

More preferably, monospecific antibodies of the present invention bind strongly to one or more *M. tuberculosis* strains with essentially no binding, under the same conditions, to *M. avium.* More preferably, monospecific antibodies of the present invention bind strongly to one or more *M. tuberculosis* strains with essentially no binding, under the same conditions, to any one of *M. avium, M. intracellulare, M. gordonae, M. fortuitum, M. smegmatis, M. szulgai, M. xenopi, M. phlei, M. terrae, M. gastri, M. scrofulaceum, M. kansasii* and *M. chelonae.* More preferably, monospecific antibodies of the present invention bind strongly to one or more *M. tuberculosis* strains with essentially no binding, under the same conditions, to any combination of *M. avium, M. intracellulare. M. gordonae, M. fortuitum, M. smegmatis, M. szulgai, M. xenopi, M. phlei, M. terrae, M. gastri, M. scrofulaceum, M. kansasii* and *M. chelonae.*

As used herein, the term 'essentially no binding' means that, in ELISA assays as described in Example 4 herein, the optical density (OD) reading at 450nM is OD $\leq$ 100 milli-A. As used herein, the term 'little binding' means that, in ELISA assays as described in Example 4 herein, the optical density (OD) reading at 450nM is 100

milli-A ≤ OD ≤ 300 milli-A. As used herein, the term 'bind strongly to' means that, in ELISA assays as described in Example 4 herein, the optical density (OD) reading at 450nM is OD ≥ 300 milli-A.

A. Antibodies and Cell Lines

Antibodies which may be used to carry out the present invention include (a) monoclonal antibody Mtb-b5.3.5, (b) monoclonal antibodies which bind to the 65 kilodalton protein (or more preferably the epitope) bound by monoclonal antibody Mtb-b5.3.5, and (c) fragments of (a) or (b) above which bind to the antigen (or more preferably the epitope) bound by monoclonal antibody Mtb-b5.3.5. Such antibodies and antibody fragments may be produced by a variety of techniques, as discussed below. The monoclonal antibody Mtb-b5.3.5 was developed by Salman H. Siddiqi, A.F. Raisur Rahman, and Richard T. Root.

The term "antibodies" as used herein refers to all types of immunoglobulins, including IgG, IgM, IgA, IgD, and IgE. Of these, IgG is particularly preferred. The antibodies may be of any species of origin, including (for example), mouse, rat, rabbit, horse, or human, or may be chimeric antibodies. *See, e.g.*, M. Walker et al., *Molec. Immunol.*, **26**, 403 (1989). The antibodies may be recombinant monoclonal antibodies produced according to the methods disclosed in Reading, U.S. Patent No. 4,474,893, or Cabilly et al., U.S. Patent No. 4,816,567. The antibodies may also be chemically constructed by specific antibodies made according to the method disclosed in Segel et al., U.S. Patent No. 4,676,980.

Monoclonal antibodies other than Mtb-b5.3.5 which bind the antigen (or more preferably the epitope) bound by the Mtb-b5.3.5 antibody may be obtained in accordance with known techniques. For example, cells identified as carrying the antigen bound by Mtb-b5.3.5 can be washed with

an aqueous solution containing a detergent to remove the antigen therefrom, the various fractions in the solution separated by chromatography (e.g., high performance liquid chromatography), and the fraction containing the antigen identified by its ability to bind the Mtb-b5.3.5 antibody. In the alternative, the Mtb-b5.3.5 antibody may be immobilized on a solid support to provide an affinity chromatography column, a sample of proteins extracted from *M. tuberculosis* cell walls passed through the column, antigen bound to the Mtb-b5.3.5 antibody eluted from the column, and the eluted antigen used to produce an antibody.

Antibodies which bind to the epitope (i.e., the specific binding site) bound by Mtb-b5.3.5 can be identified in accordance with known techniques, such as their ability to compete with labelled Mtb-b5.3.5 antibody in a competitive binding assay.

As used herein, the term antibody includes antibody fragments such as, for example, Fab, $F(ab')_2$, and Fv fragments, and the corresponding fragments obtained from antibodies other than IgG. Such fragments can be produced by known techniques. Monoclonal Fab fragments may be produced in *Escherichia coli* by recombinant techniques known to those skilled in the art. *See, e.g.*, Huse, *Science* **246**, 1275-81 (1989).

Monoclonal antibodies of the present invention may be produced in a hybridoma cell line according to the technique of Kohler and Milstein, *Nature*, **265**, 495-97 (1975). For example, a solution containing the appropriate antigen may be injected into a mouse and, after a sufficient time, the mouse sacrificed and spleen cells obtained. The spleen cells are then immortalized by fusing them with myeloma cells or with lymphoma cells, typically in the presence of polyethylene glycol, to produce hybridoma cells. The hybridoma cells are then grown in a

suitable medium and the supernatant screened for monoclonal antibodies having the desired specificity as given herein. Accordingly, a further aspect of the present invention is to provide hybridomas which produce antibodies against an antigen found on *M. tuberculosis* cell surface.

As used herein, the term antibodies includes monospecific polyclonal antibodies (e.g., antibodies of polyclonal origin which bind to a single epitope), the monospecific polyclonal antibodies directed to the same epitope as is bound by Mtb-b5.3.5. Polyclonal antibodies to the 65 kilodalton protein bound by Mtb-b5.3.5 may be produced in accordance with techniques known in the art. To obtain monospecific polyclonal antibodies directed to the epitope bound by Mtb-b5.3.5, polyclonal antibodies to the 65 kilodalton protein may be screened using a peptide consisting essentially of the epitope bound by Mtb-b5.3.5. Monospecific polyclonal antibodies may also be obtained by screening polyclonal antibodies using said 65 kilodalton protein; the 65 kilodalton protein is immobilized and saturated with Mtb-b5.3.5 (or another monoclonal antibody which binds the epitope bound by Mtb-b5.3.5), and then saturated with the polyclonal antibody, thus leaving unbound only the polyclonal antibodies specific for the epitope of Mtb-b5.3.5.

The hybridoma cell line Mtb-b5.3.5, which produces monoclonal antibody Mtb-b5.3.5, was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA, in accordance with the provisions of the Budapest Treaty on 14 December 1993, and has been assigned ATCC Accession Number **HB 11500.**

B. Subjects

The method disclosed herein may be used with specimens from subjects suspected of carrying the *M. tuberculosis* organism and specimens from subjects who have not been

previously diagnosed as having an *M. tuberculosis* infection.

The methods disclosed herein are applicable to testing for pulmonary tuberculosis, miliary (disseminated) tuberculosis, tuberculous meningitis, genitourinary tuberculosis (including renal tuberculosis), tuberculosis of bones and joints, and tuberculosis of other organs such as the pericardium, gastrointestinal tract, and adrenal glands. Specimens taken from the subject are used to initiate culture growth, and the culture growth is assayed for the presence of *M. tuberculosis*. Specimens taken from human subjects for use in preparing cultures for use in the methods disclosed herein are generally biological fluids such as sputum, blood plasma, bronchial secretions, gastric secretions, urine, cerebrospinal fluid, serous effusion, ascites fluid or pus from an abscess or sinus. In the alternative, the specimen may be a tissue biopsy sample, such as from kidney, lymph node, or other organ. The appropriate selection of the biological specimen and appropriate culture techniques will be readily apparent to one skilled in the art. Specimens taken from subjects may also be assayed directly (i.e., assaying the specimen itself for the presence of *M. tuberculosis*, rather than assaying the growth cultured from the specimen).

In a particular embodiment of the methods described herein, the subject from whom the specimen is taken has not been previously diagnosed as harboring an *M. tuberculosis* infection. In another embodiment, the subject from which the specimen is taken has been previously diagnosed as carrying *M. tuberculosis* and possibly has already undergone treatment thereof. For example, specimens may be collected from subjects prior to initiating therapy and at points subsequent thereto to assess the effects of the therapy (*see* Mitchison, *Am. Rev. Resp. Dis.*, 1062-1063 (April 1993)).

C. Immunoassay Formats

The monoclonal antibody of the present invention may be utilized in any immunoassay format known in the art as suitable for use with monoclonal antibodies. Such assays include, but are not limited to, latex agglutination assays, solid phase immunoassays, enzyme linked immunosorbent assays (ELISA), and dip stick assays. A preferred immunoassay format is a "flow through device" utilizing the antibody of the present invention. As used herein, a flow through device refers to immunoassay devices in which the monoclonal antibody is placed on a physical support able to trap bacteria while allowing liquid phases to pass therethrough. Such physical supports include filtering means, such as filter paper, which allows liquid phases of the assay to pass through the filter while retaining *M. tuberculosis*.

Immunoassays carried out in accordance with the present invention may be homogenous assays or heterogenous immunological assays. In a homogenous assay the immunological reaction usually involves the specific antibody (e.g., Mtb-b5.3.5), a labeled analyte, and the sample of interest. The signal arising from the label is modified, directly or indirectly, upon the binding of the binding pair consisting of the monoclonal antibody and the labeled analyte. Both the immunological binding of the monoclonal antibody and the analyte of interest in the sample and the detection of the extent thereof, are carried out in a homogenous solution. Immunochemical labels which may be employed to detect the binding pair include free radicals, radioisotopes, fluorescent dyes, enzymes, bacteriophages, coenzymes, and so forth.

In a heterogeneous assay approach, the reagents are usually the specimen, the antibody of the invention (e.g., Mtb-b5.3.5), and means for producing a detectable signal indicating the binding of the binding pair consisting of

the monoclonal antibody and the analyte of interest contained in the sample. Similar specimens as described above may be used. The antibody is generally placed or immobilized on a support (such as a bead, plate, filter or slide), and contacted with the specimen suspected of containing the antigen in a liquid phase. The support is then separated from the liquid phase and the support phase is examined for a detectable signal employing means for producing such signal. The signal is related to the binding of the monoclonal antibody with analyte present in the specimen. Means for producing a detectable signal include the use of detectable groups such as radioactive labels, fluorescent labels, enzyme labels, and so forth. For example, if the antigen to be detected contains a second binding site, an antibody which binds to that site can be conjugated to a detectable group and added to the liquid phase reaction solution before the separation step. The presence of the detectable group on the solid support indicates the presence of the antigen of interest in the test sample. Exemplary of heterogenous immunoassays are the radioimmunoassay, immunofluorescence methods, enzyme-linked immunoassays, luminescent immunoassays and the like.

Those skilled in the art will be familiar with numerous specific immunoassay formats and variations thereof which may be useful for carrying out the method disclosed herein. *See generally*, E. Maggio, *Enzyme-Immunoassay*, (1980)(CRC Press, Inc., Boca Raton, FL): *see also* U.S. Patent No. 4,727,022 to Skold et al., titled "Methods for Modulating Ligand-Receptor Interactions and their Application", U.S. Patent No. 4,659,678 to Forrest et al. titled "Immunoassay of Antigens", U.S. Patent No. 4,376,110 to David et al., titled "Immunometric Assays Using Monoclonal Antibodies", U.S. Patent No. 4,275,149 to Litman et al., titled "Macromolecular Environment Control

in Specific Receptor Assays", U.S. Patent No. 4,233,402 to Maggio et al., titled "Reagents and Method Employing Channeling", and U.S. Patent No. 4,230,767 to Boguslaski et al., titled "Heterogeneous Specific Binding Assay Employing a Coenzyme as Label." Applicants specifically intend that the disclosures of all U.S. Patent references cited herein be incorporated herein in their entirety. Monoclonal antibodies as described herein may be used in a "two-site" or "sandwich" assay. While a single cell line may serve as a source for both the labeled monoclonal antibody and the bound monoclonal antibody in a sandwich assay, it is understood by those skilled in the art that such labeled and bound monoclonal antibodies used in a sandwich assay must bind to the analyte at separate sites, such that the binding of the labeled monoclonal antibody does not interfere with the binding of the analyte and the bound monoclonal antibody. Exemplary assays are described in U.S. Patent No. 4,376,110.

Antibodies described herein may be supported on a solid support suitable for a diagnostic assay (such as beads, plates, slides or wells formed from materials such as latex, polystyrene; or filters, sheets, membranes, test strips, dipsticks, cards or the like formed from materials such as nitrocellulose) in accordance with known techniques. Antibodies as described herein may be conjugated to detectable groups such as radiolabels (e.g., ^{35}S, ^{125}I, ^{131}I), enzyme labels (e.g., horseradish peroxidase, alkaline phosphates), and fluorescent labels (e.g., fluorescein) in accordance with known techniques.

Detectable groups for use in carrying out the methods of the present invention include those detectable groups which are visible without further treatment when bound to the monoclonal antibody, the analyte, or the binding pair consisting of the monoclonal antibody and the analyte. Such visible detectable groups include, for example, sacks

containing a dye or some other material which is visible without lysing of the sack; such sacks include liposomes (single walled or multi-lamellar) and microcapsules (for example polymer microcapsules); (*see, e.g.*, U.S. Patent Nos. 4,703,017, 4,920,046, incorporated herein by reference in their entirety).

Diagnostic kits for carrying out the methods disclosed above may be produced in a number of ways. In one embodiment, the diagnostic kit comprises (a) an antibody of the invention (e.g., Mtb-b5.3.5) and (b) a second antibody conjugated to a detectable group. The reagents may also include ancillary agents such as buffering agents and protein stabilizing agents, e.g., polysaccharides and the like. The diagnostic kit may further include, where necessary, other members of the signal-producing system of which system the detectable group is a member (e.g., enzyme substrates), agents for reducing background interference in a test, control reagents, apparatus for conducting a test, and the like. A second embodiment of a test kit comprises (a) an antibody as described herein, and (b) a specific binding partner for the antibody conjugated to a detectable group. Ancillary agents as described above may likewise be included. The test kit may be packaged in any suitable manner, typically with all elements in a single container along with a sheet of printed instructions for carrying out the test.

In accordance with a preferred embodiment of the present invention, the test device used in carrying out the assay of the present invention is a flow through solid phase assay device as described in U.S. Patent Nos. 4,920,046, 5,073,340, 5,185,127 and 5,204,061 (applicants intend the disclosures of all U.S. patents cited herein to be incorporated herein in their entirety). Such assay devices may be used in any flow through immunoassay procedure including competitive and sandwich assays; the

end point may be color development visible to the naked eye such that no optical instruments are required to ascertain the test results. Such color development may be achieved using a monoclonal antibody supported on a test area, the monoclonal antibody capable of forming a binding pair with the analyte of interest contained in the sample, and a tracer which binds to the binding pair, wherein the bound tracer is visible to the naked eye without further treatment. Such tracers may include, for example, liposomes and microcapsules containing a visible dye (*see, e.g.*, U.S. Patent No. 4,703,017).

The assays of the present invention are suited to being carried out using samples of cultures grown on solid culture media. Suitable solid media includes egg-based media as well as agar-based media such as, for example, Lowenstein-Jensen (LJ media, available from BBL and Diffco), Middlebrook 7H 10 (BBL), Middlebrook 7H 11 (BBL), and Ogawa medium.

Assays of the present invention may also be carried out using clinical specimens directly (i.e., assaying the specimen itself for the presence of *M. tuberculosis*, rather than assaying the growth cultured from the specimen). Clinical specimens assayed directly may include, but are not limited to, sputum, bronchial secretions, blood plasma, gastric secretions, urine, cerebrospinal fluid, serous effusion, ascites fluid or pus from an abscess or sinus. In the alternative, the sample to be tested may be prepared from a tissue biopsy specimen, such as from a kidney, lymph node, or other organ. Any suitable assay device or format may be used. When clinical specimens are used in a flow-through assay device, the specimens must have physical characteristics such that the specimen can be washed through the physical support. Treatment of clinical specimens (e.g., to reduce the viscosity thereof) may be required to achieve proper flow characteristics. Such

treatments may include liquefying, diluting, centrifuging, and extracting steps performed on the specimen, and may further include additional steps as are known in the art.

As used herein, Mtb-b5, Mtb-b5.3 and Mtb-b5.3.5 designate the same antibody produced by different subclones of cells. The designations indicate the subclone origin of the monoclonal antibody.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, ml means milliliter, µl means microliter, µg means microgram, µ means micron, °C means degrees Centigrade, OD means optical density, M means molar, nM means nanometer, ELISA means enzyme linked immunosorbent assay, PBS means phosphate buffered saline; and HRP means horseradish peroxidase.

EXAMPLE 1

Antigen Preparation

Filtrate proteins for use in the following Examples were produced by growing bacteria in protein-free medium (Proskauer-Beck) for six weeks, then filtering to remove cells. The filtrate was sterilized by treating at 60°C for four hours. Proteins were quantitated using the Pierce BCA assay (see U.S. Patent No. 4,839,295). Thimerosal was added as a preservative to 0.02%.

Bacterial lysate for use in the following Examples was produced by grinding whole, heat killed bacteria in a tissue grinder for 4-5 minutes in phosphate buffered saline. Thimerosal was added as a preservative to 0.02%.

EXAMPLE 2

Mycobacterial Strains

The bacterial strains used for hybridoma production were *Mycobacterium tuberculosis* strains Va3, Va7, 351, and

H37Rv. *M. tuberculosis* H37Rv is available from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, ATCC catalog number 25618.

Additional bacterial strains used in testing the sensitivity and specificity of monoclonal antibodies were: *M. avium* (strains 33, 61, 39); *M. kansasii* (strains 714, 7, 711); *M. scrofulaceum* (1004, 78, 9); *M. gordonae* (strains 1104, 1102, 10); *M. fortuitum* (strains 2801, 16, 2803); *M. chelonae* (strains 3001, 18, 2904); *M. intracellulare* (strains 1708, 40, 1701); *M. smegmatis* (strains 17, 2401, 2403); *M. szulgai* (strains 11, 1203, 1205); *M. xenopi* (strains 1902, 1901, 1905); *M. phlei* (strains 2101, 2104, 15); *M. terrae* (strains 13, 1504, 1505); and *M. gastri* (strains 1305, 1306, 1312).

EXAMPLE 3

Immunization and Production of Hybridomas

Antibody-producing hybridomas were generated according to a modification of the original method of Kohler and Milstein, *Nature*, 256, 495-96 (1975). At week 0 BALBc/AnCrl mice were immunized intraperitoneally with a single dose of 100 µl of complete Freund's adjuvant, then at four weeks with a single dose of 22µg total protein of filtrate proteins and bacterial lysate (*M. tuberculosis* H37Rv) in water, prepared as described in Example 1, above.

At six weeks mice were injected intraperitoneally with 50µg of bacterial lysate (*M. tuberculosis* H37Rv). At 40 weeks mice were injected intraperitoneally with 50µg heat killed mixed tuberculosis cells in water, the mixed tuberculosis cells consisting of equal quantities of the following *Mycobacterium tuberculosis* strains: Va3, Va7, 351, and H37Rv. At 51 weeks, mice were injected

intraperitoneally and in the foot pads with 50μg of *M. tuberculosis* strain H37Rv culture filtrate and 50μg of H37Rv lysate in water. Animals were sacrificed four days after the last injection and the spleen and popliteal and inguinal lymph nodes were removed for fusion.

Fusion was accomplished using standard polyethylene glycol (PEG) technique. *See* Fazekas et al., *J. Immunol. Meth.* 35, 1-21 (1980). To produce hybridoma cells, the spleen cells and lymph node cells were fused with murine BALB/C line P3/X63-Ag8 myeloma cells (ATCC CRL 1580) in the presence of polyethylene glycol in accordance with the method described in Fazekas et al.

For the fusion, 1,920 wells were used. Each well contained 0.2ml of DMEM medium with 10% fetal calf serum, HMT additive (Sigma #H8016) and Ewing Sarcoma Growth Factor (ICN Biochemicals) at a 1:40 dilution. After seven days of culture, 150μl of media from each well was withdrawn and replaced with 200μl of DMEM, 10% fetal calf serum, with HT additive (Sigma #H0137). Cells were cultured for another seven days before beginning screening.

EXAMPLE 4

Screening of Hybridoma Cell Cultures

Initial reactivity screening of hybridoma cell culture supernatants was performed by ELISA, using whole heat killed cells and filtrate proteins of *Mycobacterium tuberculosis* strain H37Rv prepared as described in Example 1, above. *M. tuberculosis* cells were adhered to polystyrene plates by drying 50μl of a solution of *M. tuberculosis* cells (OD_{600}) in 0.15M sodium bicarbonate (pH=9.5) in the wells. The wells were blocked with a 100 μl solution of 10% normal goat serum in PBS for one hour. Filtrate proteins were diluted to 10μg/ml in the bicarbonate buffer, and 50μl/well were distributed.

The screening process consisted of transferring 50μl of media from a growing well to a cell-coated well, and 50μl of media to a filtrate-coated well. The wells were incubated for 1 hour at 37°C. The wells were then washed with 100μl of PBS-Tween, and 50 μl of goat anti-mouse IgGAM-HRP (Cappel, 55556) diluted 1:6,000 in PBS-Tween was added. Wells were incubated for 1 hour at 37°C. The plates were washed six times with 100 μl of PBS-Tween, and 50 μl of TMB substrate solution (Moss, TMBE-1000) was added for 10 minutes. Color development was stopped by adding 100 μl of 2M phosphoric acid, and optical density (OD) was read at 450nM. Clones showing an OD≥ 1,000 milli-A for cells, or OD ≥ 300 milli-A for filtrate proteins were selected for further development.

Of the 1,920 wells cultured, 559 were screened and 69 were selected for cloning. These 69 hybrids were cloned and cultured for the production of antibody-containing supernatant. Cells were cloned by the standard limiting dilution technique in DMEM, 10% fetal calf serum, with insulin, transferrin and selenium (ITS, Collaborative Research) added. At confluent growth the wells were tested by ELISA (as described above) for antibody expression.

EXAMPLE 5

Antibody Production and Purification

Ascites production. Ascites production was carried out by implanting 1 x 10^6 Mtb-b5.3.5 clone cells into the peritoneal cavity of BALB/C mice primed two weeks earlier with 0.5 ml Pristane (Aldrich Chem.). Ascites fluid was collected as produced, centrifuged to pellet cells, and the supernatant stored frozen.

Production in media. Cells were cultured in 30 ml of DMEM with 10% fetal calf serum in T-175 flasks until

confluent. Cells were pelleted by centrifugation and discarded. The supernatant was stored frozen.

Purification. Frozen supernatant samples were thawed (and ascites-produced supernatant was also diluted 1:4 into PBS). Ascites fluid was delipidated by extraction with 25% volume of 1,1,2-trichloro-1,2,2-trifluoroethane. This was filtered through a .22μ filter and applied to a Protein-G (Pharmacia) column (2.5 x 10 cm) at a flow rate of 1 ml/minute. PBS was used to wash non-bound proteins from the column, at 2 ml/min. Elution of the antibodies was via 10 ml of 0.5 M sodium acetate (pH=3.0). Fractions containing antibody were monitored via UV photometer, collected and immediately neutralized with ammonium hydroxide. This was dialyzed against a 1000x volume of PBS overnight. Antibody was quantitated by OD_{280} using an $\epsilon^{1mg/ml}=1.35$, preserved with 0.02% thimerosal and stored at 4°C.

EXAMPLE 6

Specificity of Monoclonal Antibody Mtb-b5.3

The 69 candidate monoclonals were subjected to specificity testing by the ELISA assay (as described in Example 4, above, using adsorbed mycobacterium) using a variety of mycobacteria species and strains. Mtb-b5 was found to bind to *M. tuberculosis* with little binding to non-tuberculosis strains as compared to other monoclonal antibody candidates. Results are shown in **FIG. 1**, where OD means optical density (milli-A units); M.tb H37Rv means *Mycobacterium tuberculosis* strain H37Rv; M.av means *M. avium*; M.sc means *M. scrofulaceum*; M.go means *M. gordonae*; M.fo means *M. fortuitum*; M.ch means *M. chelonae*; M.in means *M. intracellulare*; M.ka means *M. kansasii*.

Clone Mtb-b5 was subcloned, and subclone Mtb-b5.3 was tested for specificity. Results are shown in **FIGS. 2A** and

2B, where each square of the grid represent testing of the subclone against a particular strain of mycobacteria as indicated by **Table 1**. **Figure 2A** shows specificity testing of clone Mtb-b5.3 obtained from spent media on a variety of *Mycobacterium* species and strains; Figure 2B shows specificity testing of clone Mtb-b5.3 obtained from ascites on a variety of *Mycobacterium* species and strains. OD means optical density (milli-A units).

TABLE 1

Mycobacterial strains correlated to results as shown in Figures 2A, 2B, and 2C.

	COLUMN			
	NMS	ka7	ph15	sc9	12
	go10	ka714	ph2104	sc78	11
	go1102	ka711	ph2101	sc1004	10
	go1104	in1701	xe1905	av39	9
	BCG34	in40	xe1901	av61	8
	Va7	ch1708	xe1902	av33	7
	Va3	ch2904	sz1205	ga1312	6
	351	ch18	s1203	ga1306	5
	350	fo3001	sz11	ga1305	4
	361	fo2803	sm2403	te1505	3
	H37	fo16	sm2401	te1504	2
	Control	fo2801	sm17	te13	1
ROW:	A	B	C	D	

The final choice of monoclonal was based on the highest ratio of binding to *M. tuberculosis* strains with respect to the non-tuberculosis strains. Mtb-b5.3 consistently showed the lowest cross-reactions.

Clone Mtb-b5.3 was re-cloned to select for a high producing sub-clone, via the standard limiting dilution procedure. The high-producing sub-clone designated Mtb-b5.3.5 was selected for further development.

Subclone Mtb-b5.3.5 obtained from ascites was subjected to specificity testing using the ELISA assay as described in Example 4, above, using a variety of mycobacterial species and strains. Results are shown in **FIG. 2C**; the legend for the graph is the same as in FIGS. 2A and 2B, except that square A3 = *M. tuberculosis* 361

(361); A4 = *M. tuberculosis* 350 (350); A5 = *M. tuberculosis* 351 (351); A6 = *M. tuberculosis* Va3 (Va3); A7 = *M. tuberculosis* Va7 (Va7); and A8 = *M. bovis* BCG34 (BCG34). These results show that Mtb-b5.3.5 had good *M. tuberculosis* strain response and minimal cross reactivity. Although some cross-reactivity with *M. chelonae* 2904 appears in the specific assay reported (**FIG. 2C**), no similar cross-reaction occurred in further testing using prototype flow-through assay devices (see Examples 8 and 9) and *M. chelonae* from stock or clinical isolates (data not shown).

EXAMPLE 7

Monoclonal Antibody Mtb-b5.3.5 Binds to a 65 Kilodalton Protein

To determine the bacterial protein to which Mtb-b5.3.5 binds, *Mycobacterium tuberculosis* heat-killed bacteria were pelleted by centrifugation. Approximately 50µl of packed cells were re-suspended in 1 ml PBS. This solution was subjected to ultra-sonic disruption at 100 watts for 3 minutes, then 5 minutes, with a 2 minute pause for cooling at 4°C. The resulting suspension was centrifuged for 10 minutes at 10,000 x G. The supernatant was transferred to another tube and centrifuged for 30 minutes at 30,000 x G. This pellet was re-suspended in 0.1 ml distilled water. Protein was quantitated via the Pierce BCA assay.

This suspension was diluted to 870 µg/ml in Laemmli sample buffer, and the sealed tube incubated at 100°C for 5 minutes. One microliter samples were separated via SDS electrophoresis on a 12.5% acrylamide gel, using the Pharmacia PhastGel system. Biotinylated molecular weight standards (Pierce) were also separated on the gel. The gel was electroblotted onto PVDF membrane (Millipore, Inc.) using the manufacturer's method. The membrane was blocked with 1% non-fat dried milk in PBS-Tween (PBST) for 1 hour.

The membrane was transferred to 5 ml of Monoclonal antibody Mtb-b5.3.5 diluted to 30 μg/ml in PBST and incubated at room temperature with shaking overnight. The membrane was washed three times in 5 ml of PBST for 15 minutes, then transferred to 5 ml of Goat anti-mouse IgG+IgA+IgM-HRP conjugate (Cappell) diluted 1:6000, and Avidin-HRP (Sigma) diluted to 1 μg/ml, for 2 hours at room temperature with shaking. The blot was washed 5 times with 5 ml PBST for 15 minutes each. Bands were visualized by developing with TMB membrane stain (KP Laboratories). Molecular weight was determined from interpolation of a plot of the logarithm of the molecular weights of the Pierce standards versus the migration distance. **FIG. 3.**

These results indicate that Mtb-b5.3.5 binds to a 65 kilodalton protein.

EXAMPLE 8

Monoclonal Antibody Mtb-b5.3.5 in Dipstick Assay

A dipstick format for the detection of *M. tuberculosis* was tested using monoclonal antibody Mtb-b5.3.5. Nitrocellulose membrane was glued to a plastic stick using rubber cement. Heat-killed *M. tuberculosis* (strain H37Rv) and *M. kansasii* (strain 714) cells were diluted to OD_{600}= 125 (milli-A) in PBS. Four two-fold dilutions of these solutions were made. Two microliter aliquots of the five dilutions were spotted onto different locations of the dipsticks. The dipsticks were then dried for 10 minutes at 56°C, and blocked with 10 drops of a filtered solution of 3% non-fat dried milk and 0.5% ZONYL™ FSN-100 (Du-Pont) in PBS, for 1 minute, then rinsed with PBS-Tween. The dipstick was immersed for 5 minutes in 1 ml monoclonal antibody Mtb-b5.3.5 diluted to 20μg/ml in PBS-Tween. The sticks were then rinsed three times in PBS-Tween and dried at 37°C, and staining using goat α anti-Mouse IgG liposomes

containing the marker sulforhodamine red (visible to the eye without further treatment) was carried out. (The preparation of liposomes for use as markers in assays is known in the art; *see, e.g.*, U.S. Patents No. 4,342,826 and 4,703,017.)

Staining was measured using a Gretag D183 reflectometer (standardized against a white background), and noting the magenta value. Results are shown in **FIG. 4**, where the solid bar represents *M. kansasii* and the striped bar represents *M. tuberculosis*. These results show that cross reactivity with *M. kansasii* strain 714 is seen only at higher inoculum levels.

EXAMPLE 9

Flow-Through Solid Phase Detection Device Using MAb Mtb-b5.3.5 and Liposome Markers

The commercially available flow-through solid phase detection test kit known as Directigen™ Flu A Test (Becton, Dickinson and Co.) was modified for use with Mtb-b5.3.5 as the assay binder to detect *M. tuberculosis*. The Directigen™ Flu A Test uses a ColorPAK™ flow through assay device (Becton, Dickinson and Co.).

Sample Preparation: Two bacterial strains (*M. tuberculosis* H37Rv and *M. kansasii* 714) were used as samples and tested in separate devices. Bacterial samples were treated with nitrous acid extraction reagents as follows.

To 8.82µl of *M. tuberculosis* H37Rv cell suspension (or *M. kansasii* 714 cell suspension) 0.38 ml of Reagent A (.65 N Acetic Acid, .3% Zwittergen 310) was added. This was followed by the addition of 34µl of Reagent B, (4M sodium nitrite, .015% phenol red), which was allowed to react for 3 minutes before the addition of 34 µl Reagent C (2.6M Tris Base, .2% sodium azide). The final density of the *M.*

tuberculosis cells was OD_{600} = 0.125, six drops of which were applied to the test area of the ColorPAC™ flow through assay devices.

Test procedure: The mycobacterium solutions were applied to the test area and allowed to completely adsorb (using the ColorPAC™ assay device, the test solutions are applied to a triangular shaped area in the center of the well). Following a 1 minute wait after complete adsorption of the sample fluid, 250 µl of a filtered solution of 3% non-fat dried milk, and 0.5% ZONYL™ FSN-100 (Du-Pont) in PBS was added. Then 100 µl of Mtb-b5.3.5 diluted to 20µg/ml was added and allowed to adsorb. Then 0.5 ml of PBS-Tween was added. After complete adsorption, 1 drop of the goat anti-mouse IgG liposome (as described in Example 8) was added, and chased with 300 µl of PBS-Tween. The device was then read. The appearance of strong color was read as positive; no color was negative. A control device with no *Mycobacterium* cells was used.

Results: Tests with *Mycobacterium tuberculosis* gave a strong, clearly discernable red triangle on a white background. Tests with *M. kansasii* gave a faintly visible triangle on a white background. The control (no cells) gave a slight discoloration in the center area of the well only.

EXAMPLE 10

Flow-Through Solid Phase Detection Device Using MAb Mtb-b5.3.5 and Enzyme Markers

A flow-through solid phase assay device and test procedure for the detection of *Mycobacterium tuberculosis* was constructed using components of the Directigen™ Flu A test kit (Becton, Dickinson and Company) and the Directigen™ RSV (respiratory syncytial virus) test kit (Becton, Dickinson and Company). Both of the Directigen™

tests use a ColorPAC™ flow through test device (Becton, Dickinson and Company), which was used in the instant experiments for the detection of *M. tuberculosis* in cultures grown on solid medium.

Sample preparation: Different species and strains of *Mycobacterium* were grown on Lowenstein-Jensen (LJ) slants for three to six weeks and used as test samples. Multiple strains of each species were tested. Bacterial colonies were taken from the solid media cultures and homogenized, and 1 milliliter of the homogenized suspension was adjusted to approximately McFarland No. 0.5 - 1.0 standard. The specimen was then heated in a water bath at 95°C for 15-30 minutes. The heated suspension (1.0 ml) was then transferred to the modified ColorPAK™ test device, as described below.

Test Procedure: The assay used components of the Directigen™ Flu A and Directigen™ RSV tests, both of which use a ColorPAK™ flow through device. Monoclonal antibody Mtb-b5.3.5 was labelled with alkaline phosphatase in accordance with procedures known in the art. Buffers and reagents from the Directigen™ RSV test were used.

To conduct the assay, 1 ml of the heated sample suspension was applied to the ColorPAK™ test device, and 4 drops of reagent 1 (wash buffer, Directigen™ RSV EIA membrane blocking buffer) was added; then 4 drops of reagent 2 (Mtb-b5.3.5 conjugated to alkaline phosphatase) was added, and the sample was incubated for 2 minutes at room temperature. Then 4 drops of reagent 3 (wash buffer, Directigen™ RSV) was added; 4 drops of reagent 4 (Substrate A, Directigen™ RSV) was added, and 4 drops of reagent 5 (Substrate B, Directigen™ RSV) was added. After 5 minutes at room temperature the results were read. A colored response (purple) indicated the presence of the *M. tuberculosis* complex (*M. tuberculosis*, *M. Bovis*, *M.*

africanum, M. microti); no colored response indicated the absence of *M. tuberculosis* complex.

TABLE 2

Specificity of Mtb Detection by Mtb-b5.3.5 Against Different Species of Mycobacteria Medium: LJ Slants

Mycobacteria	No. of Strains	Results
M. tuberculosis	40	All Positive
M. gordonae	5	All Negative
M. intracellulare	10	All Negative
M. chelonae	5	All Negative
M. scrofulaceum	5	All Negative
M. avium	10	All Negative
M. xenopi	5	All Negative
M. kansasii	30	3 Positive 27 Negative

Assay done with 3-6 weeks maximum culture growth.

Results: A sample was prepared for each strain of mycobacteria and tested in a ColorPAK™ test device as described above. **TABLE 2.** All 40 strains of *M. tuberculosis* tested positive; all other species were negative except for 3 strains of *M. kansasii* which gave false positive results. Slight cross-reactivity with *M. kansasii* is not considered a problem in the clinical setting, as *M. kansasii* colonies are pigmented and *M. tuberculosis* colonies are not.

Positivity indices for sensitivity were then carried out for ten *M. tuberculosis* strains cultured on Lowenstein-Jensen slants and picked as soon as colonies could be visualized by the unaided eye. Growth from the culture was suspended in fluid adjusted to McFarland 1.0 turbidity.

1ml of each sample was loaded onto the test device as described above. The results were scored as negative (no color development), 1+ (faint but definite purple color), 2+ (purple color), 3+ (dark purple color), or 4+ (dark purple-black color).

All ten strains of *M. tuberculosis* tested gave either 1+ or 2+ results; none were negative. **TABLE 3**.

TABLE 3

Sensitivity of M.tb Detection Medium: LJ Slants Mycobacteria: M.tuberculosis		
M.tuberculosis strain	**Days**	**Results**
361	8	2+
368	14	2+
370	14	1+
373	14	2+
393	14	1+
394	14	2+
398	14	2+
399	14	2+
402	14	1+
481	14	2+

Isolated cultures grown from fresh sputum specimens from subjects with known mycobacteria infections were also tested. Seventeen specimens processed at the Maryland State Department of Health were inoculated on L-J slants. When growth was observed for the first time, several colonies were picked and a suspension was made with turbidity equal to McFarland 0.5 - 1.0 standard and the resulting cultures were assayed. All 14 known *M.*

tuberculosis infections tested positive; two known *M. kansasii* specimens and one known *M. avium-intracellulare* complex were negative. **TABLE 4.**

TABLE 4

Rapid ID on Sputum Samples Cultured on LJ (Clinical Specimens from MSHD)

Specimen No.	Results	Culture ID
4613	>4+	M.tb
4614	>4+	M.tb
4627	>4+	M.tb
4628	>4+	M.tb
4629	>4+	M.tb
4663	>4+	M.tb
4730	1+	M.tb
4759	NEG	M.kan
4792	NEG	M.kan
4665	>4+	M.tb
061	>4+	M.tb
5570	>4+	M.tb
5377	2+	M.tb
5746	2+	M.tb
5716	NEG	MAI
5231	>4+	M.tb
5230	>4+	M.tb

The device was also tested using *M. tuberculosis* colonies taken from the solid media contained on Septi-Chek AFB™ (Becton Dickinson Microbiology Systems). Media was either Lowenstein-Jensen agar or 7H10 agar. Samples were taken when colonies were first visible with the naked eye.

In all cases tested, positive reactions were noted on either the Lowenstein-Jensen agar, the 7H10 agar, or both. **TABLE 5.**

The device was also tested using mycobacterial growth on Middlebrook 7H11, Ogawa 1% and Ogawa 3% medium with similar results (data not shown).

TABLE 5

Sensitivity of M.tb Detection by Mtb-b5.3.5 Alk. Phos. Medium: Septichek AFB Slide		
M.tuberculosis	**Medium**	**Results**
H37Rv	LJ	2+
H37Rv	7H10	2+
373	LJ	2+
373	7H10	>4+
379	LJ	2+
379	7H10	1+
393	LJ	>4+
393	7H10	± TO 1+
394	LJ	± TO 1+
394	7H10	±
398	LJ	2+
398	7H10	±
399	LJ	1 TO 2+
399	7H10	>4+
548	LJ	3+
548	7H10	1+

These results indicate that the use of Mtb-b5.3.5 in a flow-through test device provides 100% sensitivity to the

strains of *M. tuberculosis* tested, and high specificity among Mycobacteria tested. Cross reactivity is limited to a few strains of *M. kansasii*.

EXAMPLE 11

Direct Testing of Clinical Specimens with Flow-Through Solid Phase Detection Device Using MAb Mtb-b5.3.5 and Enzyme Markers

Six processed, heated, smear positive sputum specimens were obtained from the Maryland State Department of Health and tested as follows. A 1 ml sample of each sputum specimen was placed directly on the flow-through solid phase detection device as described in Example 10. All six samples were detected as positive by color development in the detection device (1+ or greater) (data not shown).

Further studies were conducted using sputum specimens processed with a mixture of 4% sodium hydroxide and N-acetyl L-cysteine (NALC), as is known in the art to liquify the specimens. Use of these specimens in the flow-through detection device as described in Example 10, however, resulted in a "no-flow" problem, where the samples were too viscous to properly pass through the device. A further experiment performed with smear negative sputum specimens seeded with *M. tuberculosis* (strain H37Rv) and treated with petroleum ether also resulted in "no-flow" problem or false negative results (data not shown).

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. A monospecific antibody produced by the cell line Mtb-b5.3.5 and which binds to an epitope residing in the 65 kilodalton protein of Mycobacterium tuberculosis and mycobacteria belonging to the tuberculosis complex, wherein said monospecific antibody shows essentially no binding to Mycobacterium avium under the same conditions where said monospecific antibody binds to Mycobacterium tuberculosis.

2. A monoclonal antibody which binds to the epitope bound by the specific antibody of Claim 1.

3. A polyclonal antibody which binds to the epitope bound by the monospecific antibody of Claim 1.

4. A monospecific antibody according to Claims 1, 2, or 3 coupled to a detectable label.

5. The cell line Mtb-b5.3.5.

6. A cell line according to Claim 5, wherein said cell line consists of hybridoma cells.

7. A cell line according to Claim 6, wherein said cell line consists of hybridoma Escherichia coli cells.

8. A method of detecting the presence of Mycobacterium tuberculosis in a sample, comprising:

contacting a sample with an antibody selected from the group consisting of:

(a) a monoclonal antibody produced by the cell line Mtb-b5.3.5. and which binds to an epitope residing in 65 kilodalton protein of Mycobacterium tuberculosis and Mycobacteria belonging to the tuberculosis complex, and which shows essentially no binding to Mycobacterium avium under the same conditions where said monoclonal antibody binds to Mycobacterium tuberculosis and Mycobacteria belonging to the tuberculosis complex.

(b) a monoclonal antibody which binds to the epitope bound by the monoclonal antibody of (a), and

(c) a monospecific polyclonal antibody which binds to the epitope bound by the monoclonal antibody of (a) under conditions permitting

said antibody to bind to said protein; and then detecting the presence or absence of the binding pair consisting of said antibody and said protein;
and wherein said detecting step is carried out by homogeneous assay.

9. A method according to Claim 8, wherein said detecting step is carried out using a method selected from the group consisting of radioimmunoassay, immunofluorescence assay, luminescent immunoassay and enzyme immunoassay.

10. A kit for performing an assay for the presence of Mycobacterium tuberculosis, comprising an antibody selected from the group consisting of:

(a) a monoclonal antibody produced by the cell line Mtb-b5.3.5, and which binds to an epitope residing in 65 kilodalton protein of Mycobacterium tuberculosis and Mycobacterium belonging to the tuberculosis complex, and which shows essentially no binding to Mycobacterium avium under the same conditions where said monoclonal antibody binds to Mycobacterium tuberculosis,

(b) a monoclonal antibody which binds to the epitope bound by the monoclonal antibody of (a), and

(c) a monospecific polyclonal antibody which binds to the epitope bound by the monoclonal antibody of (a), wherein said antibody is conjugated to a detectable group.

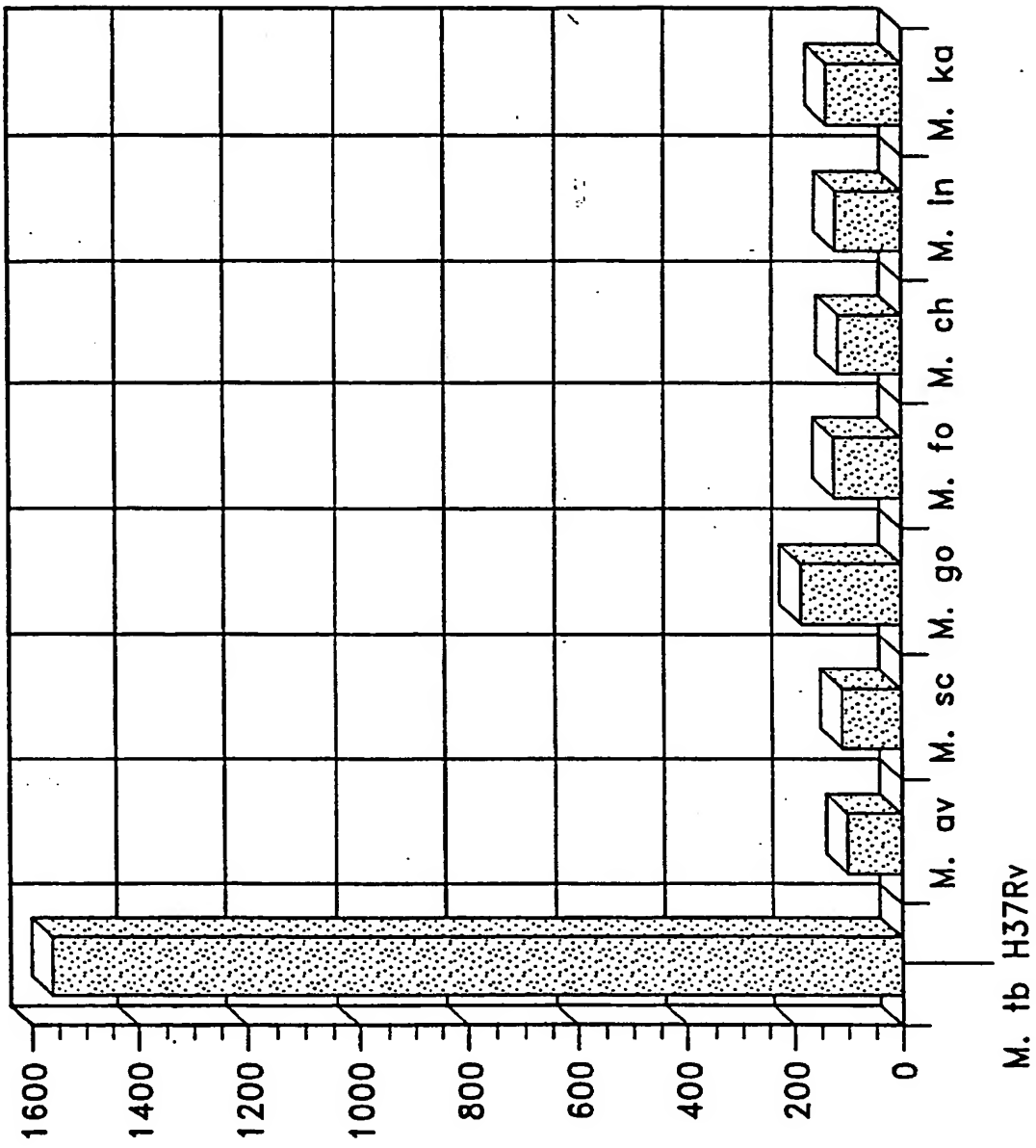


FIG-1

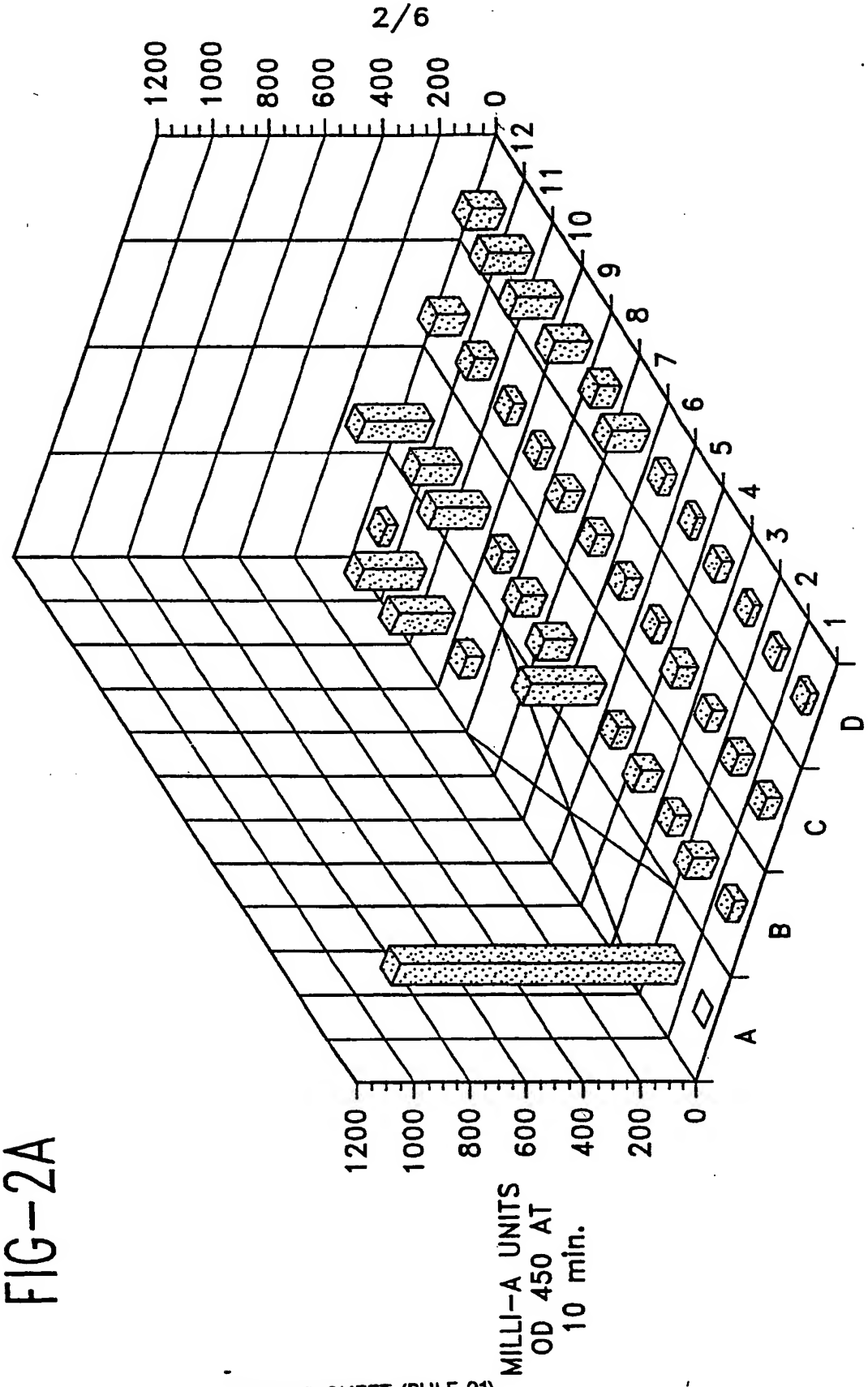


FIG-2A

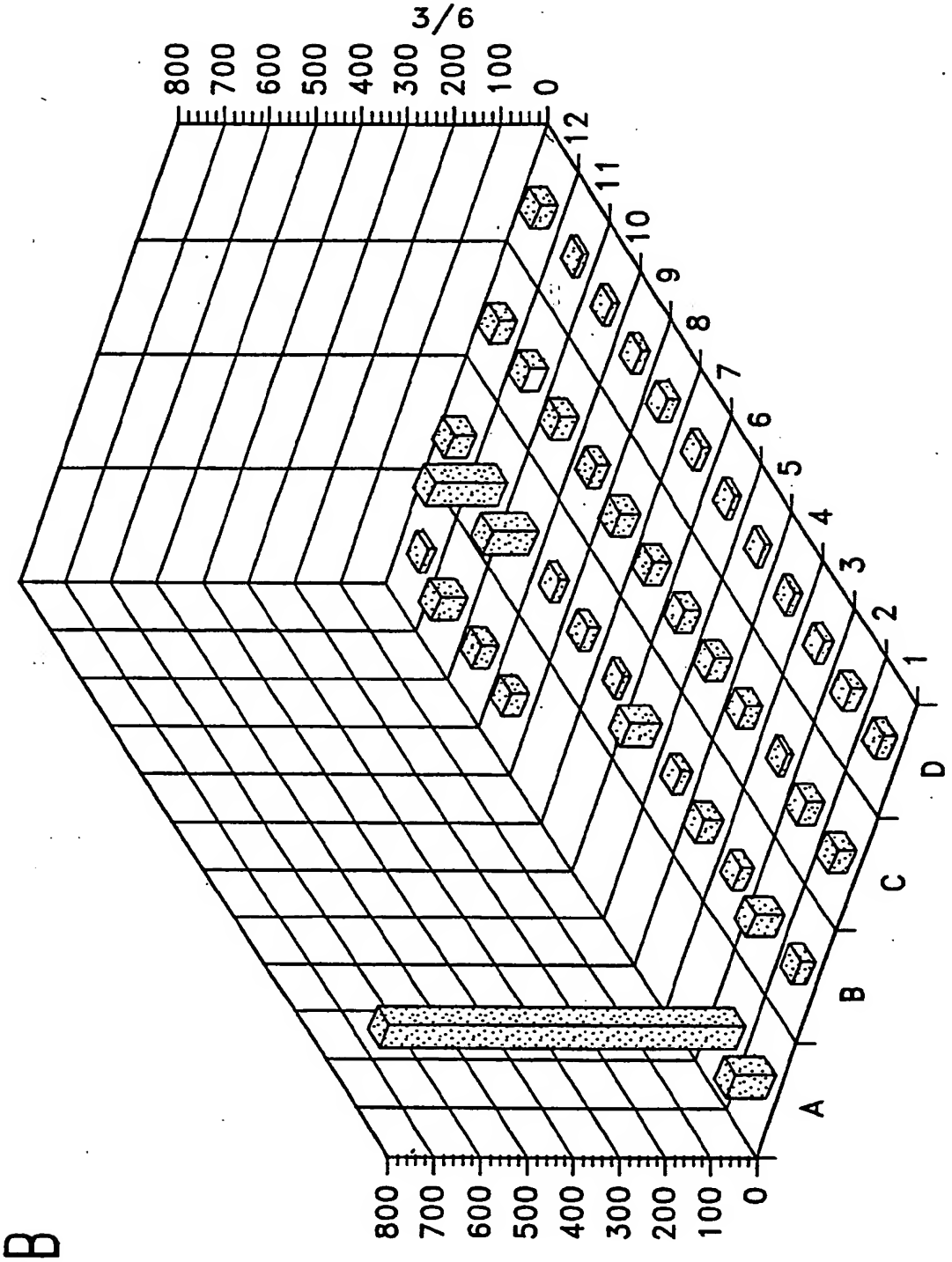
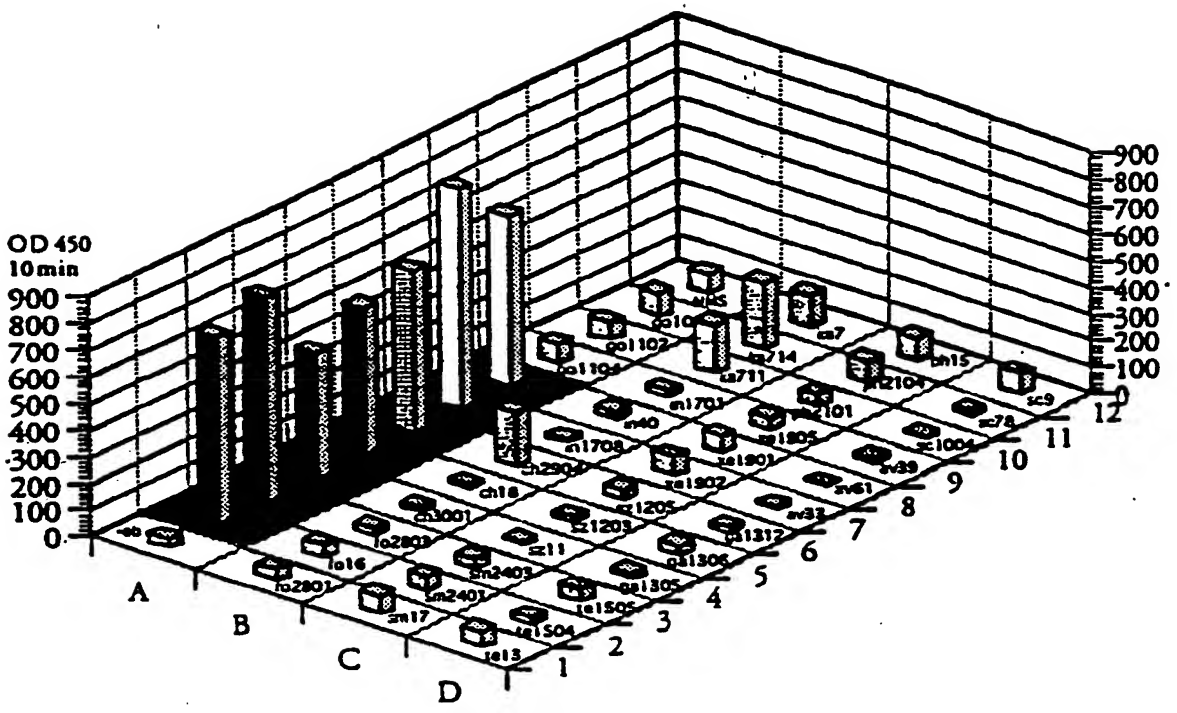


FIG-2B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10 min
900
800
700
600
500
400
300
200
100
0
A
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C
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1
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5
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10
11
12

Fig. 2C

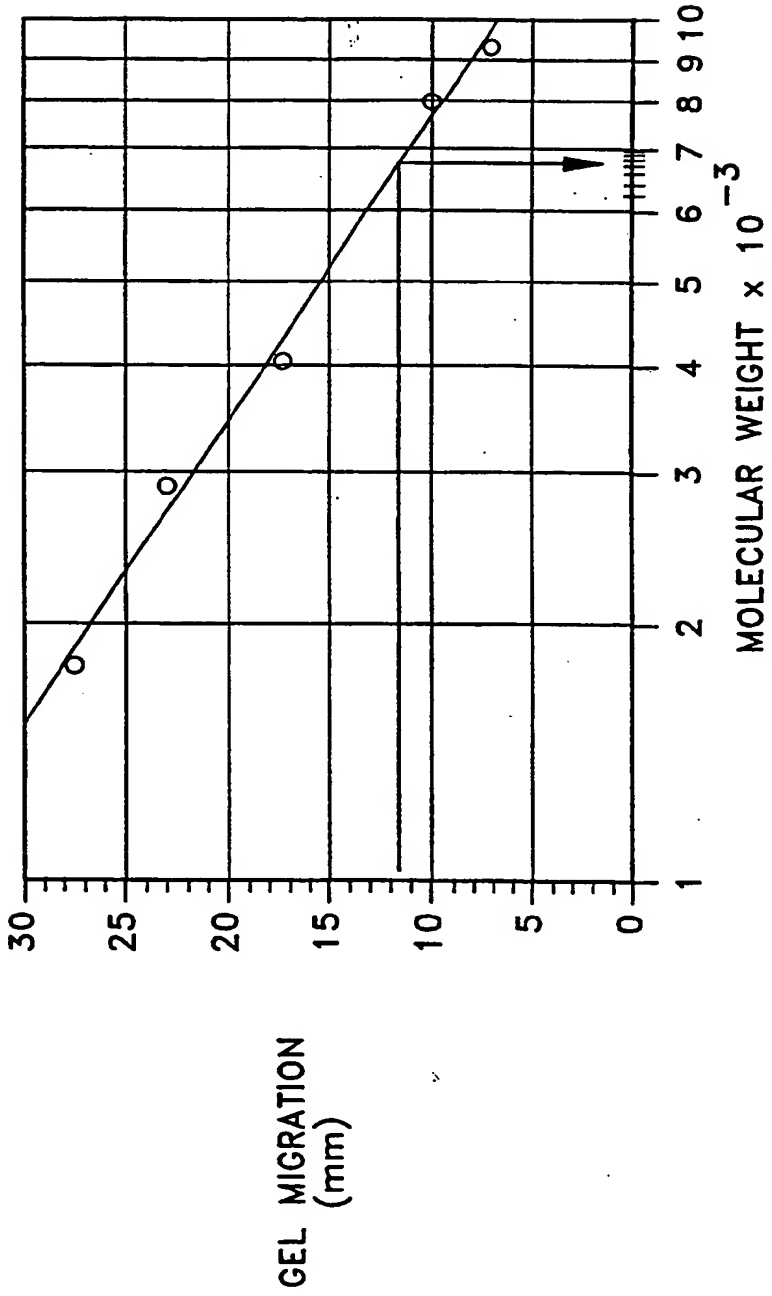

FIG-3

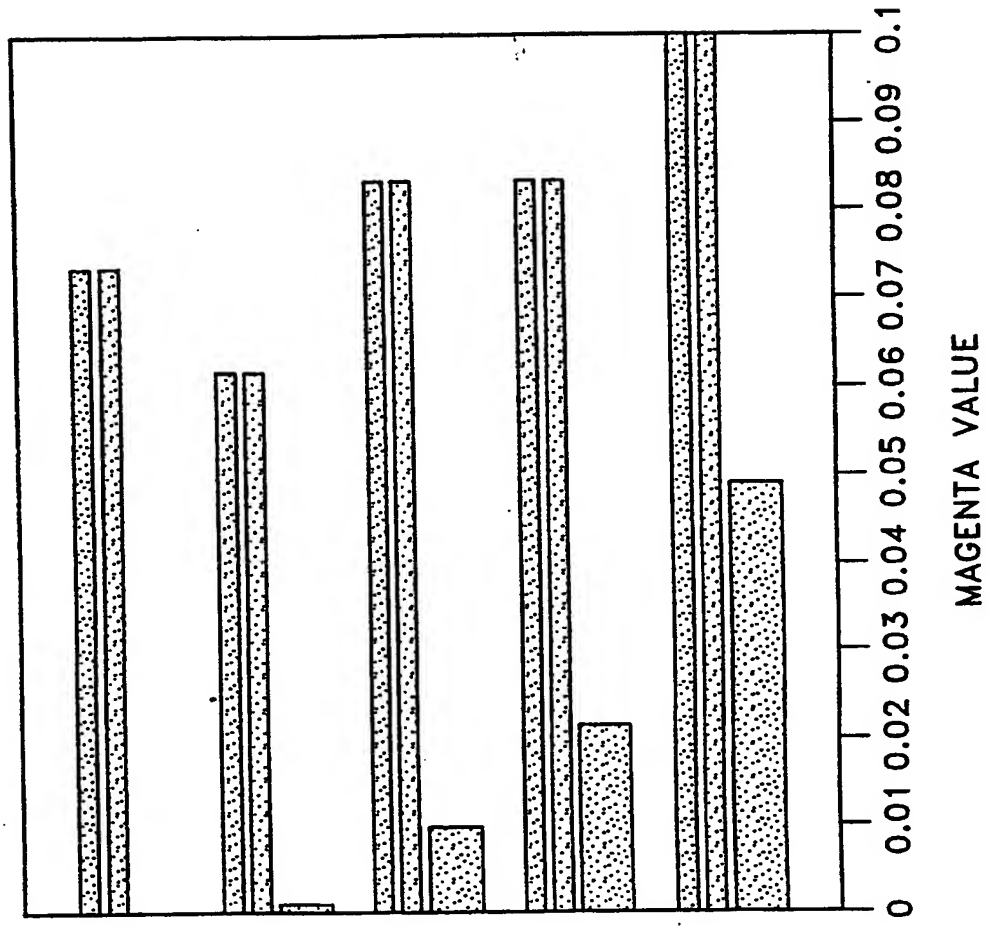

FIG-4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14685

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/7.32, 7.92, 7.94, 240.27, 252.33, 975; 436/518, 829, 808; 530/388.4, 389.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.32, 7.92, 7.94, 240.27, 252.33, 975; 436/518, 829, 808; 530/388.4, 389.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Clin. Exp. Immunol., Volume 89, issued 1992, Hajeer et al, "Monoclonal antibody epitopes of mycobacterial 65-kD heat-shock protein defined by epitope scanning", pages 115-119, see entire document.	1-3 ----- 4-10
Y	Microbiol. Immunol., Volume 35, Number 11, issued 1991, Hara et al, "Development of monoclonal antibodies reacting against Mycobacterial 65 kDa heat shock protein by using recombinant truncated products", pages 995-1007, see entire document.	5-7
Y	Science, Volume 246, issued 08 December 1989, Huse et al, "Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda", pages 1275-1281, see entire document.	5-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 1995	24 MAR 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14685

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Macario et al, "Monoclonal Antibodies Against Bacteria", Vol. 1, published 1985 by Academic Press Inc. (Florida), pages 59-90, see entire document.	4-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14685

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 35/14; C07K 14/35, 16/12, 17/100; C12N 1/22, 5/00, 5/12, 5/24; C12P 1/00; G01N 33/53, 33/543

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS; DIALOG: MEDLINE, BIOSYS, EMBASE, CA SEARCH, DERWENT WPI
SEARCH TERMS: MYCOBACTERIUM TUBERCULOSIS, IMMUNOASSAY OR ELISA OR RIA OR RADIOIMMUNOASSAY, IMMUNOGLOBULIN OR IG OR ANTIBODY, 65 KD OR 65 KILODALTON, MYCOBACTERIAL